

(19)



Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) Publication number:

0 101 348**A2**

(12)

EUROPEAN PATENT APPLICATION

(21) Application number: 83401468.0

(51) Int. Cl.³: **A 61 K 39/15**

(22) Date of filing: 18.07.83

(30) Priority: 20.07.82 US 400024

(43) Date of publication of application:
22.02.84 Bulletin 84/8(84) Designated Contracting States:
BE DE FR GB IT NL SE(71) Applicant: **UNIVERSITY OF GEORGIA RESEARCH
FOUNDATION, INC.**
612 Boyd Graduate Studies Research Center
Athens, GA 30602(US)(72) Inventor: **Page, Robert R.**
Rt 2
Winder Georgia(US)(72) Inventor: **Eldson, Caswell S.**
170 Clyde Road
Athens Georgia(US)(72) Inventor: **Gaudry, Daniel**
200 Cedar Creek Drive
Athens Georgia(US)(72) Inventor: **Villegas, Pedro**
415 Brookwood Drive
Athens Georgia(US)(74) Representative: **Lemoine, Michel et al,**
Cabinet Michel Lemoine 13 Boulevard des Batignolles
F-75008 Paris(FR)(54) **Avian proventriculitis vaccine, avian proventriculitis reovirus, process for preparing said vaccine and method of protecting a poultry animal against proventriculitis.**

(57) A process of preparing a vaccine capable of protecting poultry against avian proventriculitis which comprises : separating proventriculitis reovirus from reovirus-carrying tissue of a poultry animal diseased with proventriculitis; inoculating a cell culture capable of sustaining the growth and replication of the reovirus; extracting the virus from cellular and nonviral components, and rendering the reovirus into a form suitable for administration to poultry, yields a vaccine and an immunization method.

EP 0 101 348 A2

Express Mail: EV 630723817 US
Applicant: van de ZANDE et al.
Application No: 10/749,907

BEST AVAILABLE COPY

AVIAN PROVENTRICULIST VACCINE, AVIAN PROVENTRICULITIS REOVIRUS,
PROCESS FOR PREPARING SAID VACCINE AND METHOD OF PROTECTING A
POULTRY ANIMAL AGAINST PROVENTRICULITIS.

BACKGROUND OF THE INVENTION

Field of the Invention :

The present invention relates to the isolation of the etiologic agent of avian proventriculitis, its identification as a reovirus and the preparation of a vaccine against the disease.

Brief Description of the Prior Art :

A clinical syndrome in chickens characterized by stunted growth, poor feathering and an increase in leg weakness has been described (Kouwenhoven, V. et al, Vet. Sci. Com. 2:253-259 (1978)). In affected flocks 5-20% of the chicks exhibit growth retardation by one week of age ; an increase in lameness and very poor feather development is apparent by two weeks of age. The most prevalent clinical lesions are an enlargement of the proventriculus with areas of necrosis, hemorrhage, and catarrhal enteritis (Kouwenhoven, V. et al, Avian Pathology, 7:183-187 (1978)). Studies indicate that the syndrome can be reproduced in susceptible one-day-old chicks by direct contact with infected

chickens or by the injection of homogenized intestinal tracts from infected chickens (Vertommen, N. et al, Ibid, 9:133-142 (1980)). Biochemical examination of plasma from these chicks indicate a low plasma carotenoid level and an increase in alkaline phosphatase activity.

A number of cases with similar clinical lesions have been seen in all of the major broiler growing areas of the southeastern United States. The problem is highly farm-related and is especially severe on large farms and on farms where brooding and growing are conducted simultaneously. The first clinical signs occur when the chicks are about two weeks of age. The chicks appear chilled and do not consume as much feed as expected ; the growth rate is retarded with undigested feed passed in the droppings. Many of the droppings are covered with an orange exsudate, frequently leading to a misdiagnosis of coccidiosis. At about three weeks of age, paleness of the shank and very poor feathering are commonly seen. The clinical manifestations of this problem and the lesion patterns are extremely variable, making diagnosis of the syndrome difficult.

Post mortem lesions, like the clinical signs, present a confusing picture for the diagnostician. The lesions are not consistent from bird to bird ; however, the lesions seen most consistently include an enlarged proventriculus or an engorgement of the glands in the proventriculus. In several instances, there will be a necrosis of the proventricular glands and a small atonic gizzard. Hyperemia and hemorrhages are frequently seen in the duodenal area, and the lower portion of the GI tract will be ballooned and very friable. Perosis and femoral head necrosis are found in many of the chickens exhibiting the aforementioned lesions, and encephalomalacia is also frequently found.

The disease has been given several different names : malabsorption syndrome, pale bird syndrome, brittle bone disease, helicopter disease, and proventriculitis. The latter ("proventriculitis") will be used throughout the present specification and claims.

5 In order to further understand the nature and scope of the present invention, it is also worth describing the nature of the so-called "reoviruses". The term "reovirus" (Respiratory Enteric Orphan Virus) refers to a group of RNA viruses that infect both the respiratory and intestinal tracts, usually without producing disease (See for
10 example, Davis, B. D. et al, "Microbiology. Including Immunology and Molecular Genetics", 3rd edition, (1980) Harper & Rowe Publishers, Hagerstown, pp. 1206-1212). They include species that infect humans, birds, dogs and monkeys. They
15 appear to be ubiquitous in nature, since specific viral inhibitors (presumably antibodies) have been found in the serum of all mammals tested except the whale. Humans and many other species, (including cattle, mice and monkeys) are naturally susceptible to reoviruses.

20 Reoviruses have frequently been isolated from the feces and respiratory regions of healthy persons, as well as from patients with a variety of clinical illnesses, particularly minor upper respiratory and gastrointestinal disease. The relation of these viruses to disease is not clear. In
25 fact, Davis et al, supra, at page 1212, state :

The reovirus infections do not seem to be of great clinical importance, and further studies are needed to define their pathogenic potential. Both the respiratory and the gastrointestinal tracts may well be sources for their spread.....

30 Antibodies are also frequently found in various wild and domestic animals, but it is not known whether the animals serve as reservoirs for human infections.

Since the meager data assign a limited pathogenicity to these viruses, specific immunization procedures are not warranted.

5 This statement, coming in 1980, reflects the belief in the art that reoviruses are not involved in any serious diseases or that, at best, significant research would have to be carried out in order to define their pathogenic potential.

10 A need, therefore, continues to exist for the identification of the etiologic agent of avian proventriculitis, as well as its isolation and possible preparation of a vaccine against the disease.

SUMMARY OF THE INVENTION

15 It is therefore an object of the invention to provide a method for the purification for, and a purified form of the etiologic agent of avian proventriculitis.

It is another object of the invention to provide an avian proventriculitis vaccine.

20 It is still another object of the invention to provide a method of protecting poultry against proventriculitis.

25 These and other objects of the invention which will hereinafter become more readily apparent, have been attained by providing :

A vaccine capable of protecting poultry against proventriculitis.

30 These objects have also been attained by providing a substantially pure form of avian proventriculitis reovirus.

BRIEF DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention is based on the discovery that the etiologic agent for avian proventriculitis is a reovirus. Following this discovery, it is now possible to isolate and purify this agent, and prepare appropriate vaccines based on attenuated or unattenuated or inactivated forms of the reovirus.

The discovery that the agent is a reovirus came as a surprise, since it comes in the context of the belief in the art, presented by Davis et al, supra, that "reovirus infections (do) not seem to be of great clinical importance". Also, the preparation of a proventriculitis vaccine based on such reovirus stands in sharp contrast to the same Davis et al statement quoted previously to the effect that, given the limited pathogenicity of the reoviruses "specific immunization procedures are not warranted".

The discovery of the reovirus and the preparation of a vaccine against the disease opens the possibility of protecting poultry by wide-range immunization procedures, greatly affecting the economics of the poultry industry.

The proventriculitis virus can be isolated from the intestinal tract and visceral organs of poultry (e.g., chickens, turkeys, ducks, etc.) showing clinical signs of proventriculitis.

Tissue homogenates from visceral organs and intestinal content can be processed by grinding each sample in sterile growth medium, appropriate for the growth of the derived animal cells, but containing appropriate antibiotics such as penicillin, streptomycin or gentamycin, in order to prevent bacterial contamination. Grinding is carried out for a time and under conditions sufficient to allow the extraction of virus particles into the growth medium.

Samples are then normally filtered through a filter capable of allowing passage of the virus therethrough but not of any cells or aggregates thereof.

The filtrate is normally inoculated into confluent monolayers of pathogen-free cells capable of sustaining the growth of viruses and the replication and culture thereof. Such cells include chicken embryo kidney cells, and chicken embryo fibroblasts. The supernatant can also be inoculated in 9-11 day-old chicken embryos. The cells are grown in an appropriate growth medium, which normally contains foetal calf serum. The cells in monolayer culture are observed daily until the activity of the virus upon the cells is found. If the effect is not observed in three to five days, the plates can be frozen for three times and further passaged. At least four blind passages should be made before a sample is to be considered negative.

In samples where cytopathic effects are observed, infected cell plates are frozen a few times and cell culture fluid centrifuged under g conditions and times sufficient to separate cell fragments from viral particles, e.g., at 5,000 xg for 20 minutes. The supernatant which contains the virus is then ultracentrifuged under g conditions and times sufficient to precipitate the viral particles, e.g., at 110,000 xg for 45 minutes on an ultracentrifuge. The resulting pellet is resuspended in physiologically buffered solution, and contains the active virus.

The isolates can be identified as reoviruses on the basis of their cytopathogenicity, insensitivity to treatment with chloroform, presence of RNA, and in some cases, electron microscopy. Most isolates can be obtained after 2-3 blind passages in chicken embryo cells.

Electron microscopy reveals that the viral isolates are 68-72 nm in diameter, and icosahedrons with a double capsid shell. All of the isolates are insensitive to

both chloroform and IUDR (iododeoxyuridine), indicating that they are nonenveloped and of RNA type nucleic acids respectively. The viral strains are heat stable for up to 6 hours at 56°C and they do not hemagglutinate red blood cells (RBC's).

Inoculation of chicken embryos with active viral strains produces mortality within 1-7 days. Macroscopic lesions include thickening and necrosis of the chorioallantoic membrane, liver necrosis and hemorrhagic embryos. Microscopically, chorioallantoic membranes become inflamed, necrotic and undergo fibroblastic proliferation. Large basophilic granular inclusions are also found in fibroblast type cells of the chorioallantoic membrane in embryos inoculated with certain strains.

The availability of purified isolated avian proventriculitis virus, substantially free of cellular components and other nonviral components, allows for the preparation of proventriculitis vaccine. The vaccine can be prepared according to a number of well-known methods in the art. Thus, a vaccine can be prepared from the whole live virus or from immunologically active but nonpathogenic subcomponents thereof, such as capsids and the like obtained by splitting with enzymes or solvents. Chemically attenuated live or killed viral vaccines can also be used, for example by the treatment of virus with propiolactone, dilute formalin, ethyleneimine, halogenated hydrocarbons, and the like. These agents decrease virus pathogenicity while allowing the material to retain immunogenicity.

Another technique for attenuating the virulence of the virus is to develop avirulent or slow growing strains, or mutants incapable of sustained replication in the host. This is generally known in the art as "genetic attenuation"

and can be done by genetic manipulations or by serial passages. For example, the production of live attenuated viruses can be carried out by adapting the isolated virus to
5 cultures containing avian tissue cells and attenuation for example by 10-200 passages in such cultures, after which said viruses multiply and a vaccine is then prepared. Another method of producing live vaccine is to select and culture clones. If the infected cells are used for the pro-
10 duction of a live vaccine, it is advantageous to release the virus from the cells. Techniques for preparing vaccines are generally detailed in a publication such as "Newcastle Disease Vaccines : Their Production and Use" -Allan, W.H., J.E. Lancaster and B. Toth ; Food and Agricultural Organi-
15 zation, Rome 1978. This text is herein incorporated by reference.

The vaccines, whether live or attenuated, in their many different forms can be prepared in suspension in a manner known per se with a pharmacologically acceptable
20 vaccine carrier, such as a bioacceptable oil. It is advantageous to add thereto a stabilizer, particularly if a dry preparation is prepared by lyophilization. ~~An~~ adjuvant such as aluminium hydroxide may also be added. The stabilizing agent can be a carbohydrate such as sorbitol, mannitol,
25 starch, sucrose, dextran or glucose ; a protein like albumin or casein ; a protein containing agent like bovine serum, or skimmed milk, and a buffer such as alkaline metal phosphate.

The vaccine can be administered to poultry to prevent the same from developing proventriculitis. The poul-
30 try includes chickens, turkeys, ducks, hens, and roosters.

Immunization can occur anywhere between the time of birth to approximately 5 to 7 weeks. This will allow for the vaccine to provide adequate protection in broiler chickens which are the more severely affected by the condition.
35 However, immunity could last longer than these periods.

The vaccine can be administered, for example, by forced oral administration to individual chickens, wherein a few birds of a flock receive the vaccine and the remaining birds become vaccinated by infection. Another method is to administer the vaccine in the drinking water, by wing stabbing methods, subcutaneously, intramuscularly, by the nasal or eye drop route, etc. Most preferably, the vaccine is administered to reproductive hens, so as to provide immunity to the progeny.

A dosage of anywhere between 5×10^6 viral particles to 1.5×10^7 viral particles per administration is suitable, most preferred about 10^7 virus particles per administration.

The present methods for preparing the vaccine lead to titers of anywhere between 10^7 to $10^{8.5}$. It is, however, envisioned that the titer can be increased by concentration or by technical improvements in the methodology.

The availability of purified proventriculitis reovirus also allows for the development of immunoassay procedures for the detection of the disease in its early stages. Antibodies (IgG, IgM) against the reovirus can be generated in appropriate animals (poultry, horses, rabbits, goats, and the like), or in hybridomas (see e.g. Koprowski et al, U.S. Patent 4,196,265, or Wands et al, U.S. Patent 4,271,145 which are herein incorporated by reference). These antibodies can be used in any of the multiple immunoassay procedures currently available (see, for example T. Chard "An Introduction to Radioimmunoassay and Related Techniques", North-Holland, 1978, or Schuurs, A.H.W.M. et al, "Enzyme Immunoassay", Clin. Chim. Acta 81, 1-40 (1977), both of which are herein incorporated by reference). For example, the presence of the reovirus in

poultry samples can be detected by radioimmunoassay, enzyme immunoassay, or latex agglutination immunoassay, in physiological samples of poultry such as blood, droppings, tissue and the like. The technique utilized can be e.g. competitive, "sandwich" (forward, reverse or simultaneous),
5 double antibody, or enzyme cascade, which are all well known to those skilled in the art. It may be useful for certain techniques to prepare, by art-known methods, detectably labeled proventriculitis reovirus, such as reovirus
10 labeled with a radiolabel (I^{125} , C^{14} , H^3 , P^{32} , etc.), with an enzyme (alkaline phosphatase, peroxidase, etc.), with a fluorescent probe, and the like. The antibodies can be either in solution or immobilized, such as, for example, on the inside of tubes, on polymer or glass beads, on plastic
15 strips, and the like.

Having now generally described this invention, the same will become more readily apparent by reference to certain specific examples which are included herein for purposes of illustration only and are not intended to be limiting of the invention or any embodiment thereof.
20

Example 1Identification and Serological Differentiation
of Several Viral Strains Isolated From Chickens
with Proventriculitis

5

Materials and Methods

Source. Viral strains were isolated from the intestines from 3- to 6-week-old broilers exhibiting clinical signs of proventriculitis.

10

Procedures Used for Virus Isolation. Tissue samples were processed by grinding each sample in 15 ml sterile tryptose phosphate broth containing 100 units of penicillin and 100 micrograms of streptomycin per milliliter. The samples were then filtered through a .45 micron filter and inoculated onto confluent monolayers of specific pathogen-free (SPF) chicken embryo kidney cells (CEKC). Inoculated and uninoculated CEKC monolayers were observed daily for cytopathic effect. If the effect was not observed in 3 to 5 days, the plates were frozen-thawed three times and further passaged. At least four blind passages were made before a sample was considered negative.

15

20

25

30

Electron microscopy. Briefly, for sample preparation, the infected CEKC plates were frozen-thawed three times and cell culture fluid was centrifuged at 5,000 xg for 20 minutes. This supernatant was then ultracentrifuged at 110,000 xg for 45 minutes on a Beckman ultracentrifuge (Model L2-65B). The resulting pellet was resuspended in .1 ml of sterile phosphate buffered saline and 1 drop was allowed to dry on a coated grid. The sample was then negatively stained with 2% phosphotungstic acid pH 7.2 for 1 minute and observed with a transmission electron microscope.

Chloroform Sensitivity. The method described by Rovozzo and Burke (A Manual of Basic Virological Techniques. Prentice Hall, Inc. Englewood Cliffs, N.J., 1973) was followed, except that one aliquot of the virus suspension was
5 treated with 10% reagent grade chloroform, the other aliquot was left untreated and the tubes were centrifuged at 1,000 g for 20 minutes. Known chloroform sensitive and resistant viruses were always processed simultaneously in the same manner.

10 Iododeoxyuridine (IUDR) Inhibition and Heat Stability. IUDR inhibition and heat stability were determined following the procedure of Rovozzo and Burke, supra, utilizing CEKC as the indicator system.

Hemagglutination tests. Virus infected CEKC mono-
15 layers showing 80% cytopathic effect were frozen thawed three times and one drop of the supernatant was mixed on a clean white ceramic plate with an equal amount of 5% chicken red blood cells. The plate was gently rocked for one minute and observed for the presence of hemagglutination. Chicken
20 red blood cells and positive controls were also run in the same manner.

Pathogenicity in Embryos. SPF chicken embryos were inoculated with four of the viral isolates (approximately 10^6 PFU/embryo) by the chorioallantoic sac (CAS), chorioallantoic membrane (CAM), and yolk sac (YS) routes (5 embryos/
25 route/viral isolate). Records on mortality, macroscopic and microscopic lesions were noted.

Replication Cycle. The one-step growth curve was determined for one of the viral strains (81-5) following the procedure described by Rovozzo and Burke, supra, except that
30 the inoculum used was sufficient so that every cell in the culture was infected (10^5 TCID₅₀/plate), and the sample time was decreased to two hour intervals for a total of twenty-one hours. Chicken embryo fibroblasts were used to titrate
35 the intracellular and extracellular virus in microtiter plates (5 wells/dilution).

Cross Neutralization Studies. The beta neutralization method with a constant amount of virus and variable amount of serum was employed for all cross neutralization studies. The tests were performed in sterile flat bottom microtiter plates using SPF CEKC as the indicator system.

5 Cell and virus controls as well as known positive (homologous) and negative (SPF) sera were included in every test. The technique consisted of adding in the first row of a microtiter plate 100 microliters of a virus dilution containing 20 plaque forming units (PFU). Rows 2-11 received
10 50 microliters/well of diluted virus. The twelfth row was blanked with 50 microliters/well Hanks Balanced Salt Solution (HBSS) and served as the cell control. Twenty-five microliters of each serum sample was mixed with the 100 microliters in the wells of the first row. After discarding
15 25 microliters of the mixture, the serum was serially diluted by passing 50 microliters. The eleventh row was left as a virus control. The plates were incubated at 37°C for forty-five minutes and then approximately 2×10^5 CEKC were added to each well. After incubation at 37°C in 5% CO₂ for
20 3-4 days the plates were fixed with 95% ethanol and stained with 1% crystal violet. The titer of the serum was read as the reciprocal of the highest dilution which inhibited clearing of the monolayer. Serum samples were always run in triplicate and were repeated several times.

25 Production of Antisera. Several three to four week old SPF chickens, which were reared in Horsfall isolation units, were inoculated intraocular, intranasal and orally with approximately 10^6 PFU in 0.2 ml of cell and serum-free live virus. Two weeks later they were reinfected by the same
30 route with 0.25 ml of the same virus preparation. Blood was collected two weeks after that, centrifuged at 200 g for 10 minutes and the serum was collected and stored in 2-3 ml aliquots at -70°C.

Calculation of relatedness values. Relatedness values (R) were calculated using the formula of Archetti and Horstfall ("Persistent Antigenic Variation of Influenza A Viruses After Incomplete Neutralization in Ovo
 5 with Heterologous Immune Serum." J. of Exp. Med. 92: 441-462. 1950). The resulting R values were evaluated (Table 1) based on the work of Brooksby ("Variants and Immunity : Definitions for Serological Investigations. In :
 10 International Symposium on Foot and Mouth Disease Variants and Immunity". Lyon, France. Symposium Series Immunobiology, 1967) which interprets the R values as shown in Table 1.

15 Table 1 - Interpretation of relatedness values

	R Value (%)	Interpretation
	0-10	Serotype
20	10-32	Major subtype
	32-70	Minor subtype
	>70	Little or no

25 Results

For virus isolation, two or three blind passages were usually necessary before CPE was observed, although in some cases CPE was detected in the first passage. As early as 1-2 days post-inoculation there was syncytial formation
 30 which developed into multinucleated giant cells. By 3-5 days post-inoculation large areas of the monolayer had detached.

As seen in Table 2, the electron microscopy results revealed that the viral isolates were 68-72 nm in diameter, with icosahedral symmetry and with a double capsid
 35 shell.

All of the isolates were insensitive to both chloroform and IUDR indicating that they were non-enveloped and of RNA type nucleic acid, respectively. The viral strains were heat stable for up to six hours at 56°C and they did not hemagglutinate chicken RBC's.

Table 2. Characteristics of viral strains isolated from chickens with Proventriculitis

Characteristics	Viral Strains					
	CO ₈	43A	81-5	45	82-9	197 # 28
Electron microscopy						
- diameter (nm)	72	68-70	72	70	72	72
- icosahedron symmetry	Yes	Yes	Yes	Yes	Yes	Yes
- double capsid shell	Yes	Yes	Yes	Yes	Yes	Yes
Presence of envelope	No	No	No	No	No	No
Nucleic acid type	RNA	RNA	RNA	RNA	N.D. ^A	N.D.
Heat stability ^B	Yes	Yes	Yes	Yes	N.D.	N.D.
Hemagglutination of chicken RBC's	No	No	No	No	No	No

^A N.D. = not done

^B Six hours at 56°C

Table 3 shows the results of the inoculation of chicken embryos with four of the viral strains. Regardless of the route of inoculation, mortality was observed between one to seven days post inoculation. Embryos inoculated with strains CO₈ and 81-5 died between 3-7 days post inoculation while those embryos inoculated with strains 43A and 45 die within 3 days after inoculation.

Macroscopic lesions included thickening and necrosis of the chorioallantoic membrane, liver necrosis and hemorrhagic embryos. Microscopically the CAM were inflamed, necrotic and undergoing fibroblastic proliferation. Large, basophilic, granular inclusions were also found in fibroblast type cells of the CAM in embryos inoculated with strain 43A. Strain CO₈ produced necrosis, proliferating fibroblasts and giant cells in the liver, and also hyperplasia of the epithelial cells of the proventriculus.

Table 3. Mortality of chicken embryos after inoculation with several viral strains by three different routes

Viral Strains	Titer of inoculum PFU/0.1 ml	Route of inoculation ^A		
		CAS	CAM	Y.S.
		Days till embryo death		
CO ₈	10 ⁶	6-7	4-6	3-4
81-5	10 ^{5.4}	6	4	5
43A	10 ^{5.4}	2-3	2	3 ^B
45	10 ^{5.5}	1-3	2	3 ^C

^A CAS = chorioallantoic sac.

^B Inoculum = 10^{4.4} PFU/0.1 ml.

^C Inoculum = 10^{4.5} PFU/0.1 ml.

The results of the one step growth curve for strain 81-5 are shown in Table 4. It was found that during the first 5 hours after inoculation there was no significant rise in virus titer over the original inoculum titer. Therefore, the original inoculum titer was considered base line.

Between 5 and 7.5 hours there was a substantial increase in the intracellular virus titer while the extracellular titer remained below base level. Total virus increased rapidly between 5 to 10 hours and continued increasing at a much slower rate after that. At 7.5 hours the extracellular virus titer started to rise rapidly and continued to gradually increase after 10 hours. By 21 hours the intracellular and total virus titers had dropped slightly.

Table 4. Titer of the intracellular, extracellular and total virus obtained periodically for the determination of the one step growth curve for strain 81-5

Virus	Hours post inoculation						
	0	2	3.5.	5.	7.5.	10	21
Extracellular	$10^{3.6.A}$	$10^{4.9}$	$10^{4.9}$	$10^{4.2}$	$10^{4.4}$	$10^{6.4}$	$10^{2.4}$
Intracellular	$10^{4.8}$	$10^{4.9}$	$10^{5.2}$	$10^{5.4}$	$10^{9.3}$	$10^{9.6}$	$10^{9.1}$
Total	$10^{4.8}$	$10^{5.2}$	$10^{5.4}$	$10^{5.4}$	$10^{9.3}$	$10^{9.6}$	$10^{9.2}$

A = TCID₅₀/ml.

Geometric mean cross neutralization titers obtained with homologous and heterologous antisera are presented in Table 5. Strains 43A, 45, 81-5 and S1133 had a very low neutralization titer with the CO₈ antiserum while the homologous and heterologous titers were always of much higher values. Strain CO₈ had a very low neutralizing activity against antisera from strains 43A and 81-5 while against antisera from strain 45 and S1133 the neutralization titers were higher.

Table 5. Geometric mean cross neutralization titers
between several viral strains

5	Antigen	Antisera				
		43A	45	CO ₈	82-5	S1133
10	43A	603	757	37	905	1106
	45	1490	1120	50	-	1955
	CO ₈	40	474	269	70	190
	81-5	326	711	9	577	1902
	S1133	508	1920	22	422	905
15						

Relatedness values obtained for all strains are presented in Table 6. It was found that isolate CO₈ had an R value of 6.19 and 6.07 for strains 43A and 81-5, respectively. These values were interpreted as serologically different types. The R values for strain CO₈ compared with strains S1133 and 45 were 12.49 and 19.58, respectively. These values were interpreted as major subtype differences. Little or no difference was found/strain S1133 was compared when with strains 43A, 81-5, and 45. No difference was found among these last 3 strains.

Table 6. Relatedness values of several reovirus strains

	Strains	R Value (%)	Interpretation
5	CO ₈ + 43	6.19	Serotype
	CO ₈ + 81-5	6.07	Serotype
	CO ₈ + S1133	12.49	Major Subtype
	CO ₈ + 45	19.58	Major Subtype
10	S1133 + 43A	101.44	Little or no
	S1133 + 81-5	124.00	Little or no
	S1133 + 45	192.42	Little or no
15	81-5 + 43A	92.12	Little or no
	43A + 45	143.95	Little or no

Discussion

Viruses were not isolated from all intestinal samples processed. The physiochemical and morphological characteristics of the viruses isolated indicate that they belong to the reoviridae family.

Cross-neutralization studies indicate that the reovirus isolates belong to, at least, two different serotypes. Strain CO₈ has a serotype or major subtype difference when compared with the other reovirus strains including reovirus S1133. Broilers given viral arthritis vaccine (S1133) may not be protected against infection with viral strains similar to isolate CO₈.

Example 2Reproduction of Proventriculitis with virus
Isolates in Broiler Chickens5 Materials and Methods

 All of the trials reported herein were conducted on broiler chicks obtained from a breeder flock with a viral arthritis serum neutralization titer of 15. Proventriculitis had been seen in several farms that contained progeny from this flock. In trial 1 sixty chickens were divided into six groups of ten chicks each. Two groups each were injected at one day of age with isolate one or two and two groups were noninjected controls. In trial 2 seventy chicks were divided into seven groups of ten chicks. six groups were injected at one day of age with isolates obtained from several different farms and one group was a noninjected control. All of the birds were held in Horsfall-Bauer units with filtered air under positive pressure until trial termination. Body weights were recorded by sex and observations were made on skeletal development, feather development and the incidence of visceral lesions. Visceral tissue and bone were collected from each group for histological evaluation.

 The viruses used in these studies were isolated from the intestinal tracts and visceral organs of chickens on several farms in the southeastern United States (see Example 1).

 The feed used in both trials were a broiler starter ration manufactured by the University of Georgia Poultry Science Department. A coccidiostat was not included in any of the feeds used in these trials.

30

Results

The most common clinical feature seen in the reproduced syndrome was very irregular feather development with many broken and poorly developed feathers. The shanks were pale and there was an increase in the incidence of perosis and other leg disorders. The most commonly observed visceral lesions were an increase in pericardial fluid with an enlargement of the proventriculus and a decrease in the size of the gizzard. In many instances there was engorgement of the glands of the proventriculus without a concurrent reduction in the size of the gizzard. Lesions in the intestinal tract consisted primarily of an increase in the size of the intestinal tract with an excessive amount of mucus and hemorrhage on the mucosal surface. The intestinal tracts were very friable and easily broken. Poorly digested feed with a characteristic orange-tinged exsudate was usually found in the lower portion of the intestinal tracts.

In trial 1 there was a pronounced reduction in weight (Table 7) ; a similar, although less pronounced, effect was seen in trial 2 (Table 8).

Table 7. Weight in Grams

25	<u>Group</u>	<u>M</u>	<u>F</u>
	Controls	1235 ^a	950
	Isolate 1	1028	786
30	Isolate 2	860	799

a
Avg. wt. for both replicates, 10 chicks per replicate.

Table 8. Weight in Grams

	<u>Group</u>	<u>F</u>	<u>M</u>
5	Control	840 ^a	972
	Isolate 1	826	932
	Isolate 2	795	931
	Isolate 3	793	879
10	Isolate 4	528	944
	Isolate 5	834	924
	Isolate 6	743	889

15 ^aAv. wt. of 10 chicks in each group.

Gross visceral lesions were produced by both isolates in trial 1, with an enlargement of the proventriculus and an enlarged, friable intestinal tract being the most common lesions (Table 9). Similar lesions were produced by all six isolates used in trial 2 (Table 10).

Table 9. Gross Visceral Lesions

	<u>Heart</u>	<u>P.V.</u>	<u>Gizzard</u>	<u>Intestines</u>
25	Control	0/20	0/20	1/20
	Isolate 1	9/20 ^a	13/20	14/20
	Isolate 2	11/20	15/20	13/20

30 ^aNo. with. clinical lesions over the number posted.

Table 10. Gross Visceral Lesions

		<u>Heart</u>	<u>P.V.</u>	<u>Gizzard</u>	<u>Intestines</u>
5	Control	0/10	0/10	1/10	1/10
	Isolate 1	2/10 ^a	3/10	0/10	0/10
	Isolate 2	2/10	2/10	3/10	0/10
	Isolate 3	3/10	3/10	1/10	3/10
10	Isolate 4	4/10	3/10	2/10	2/10
	Isolate 5	4/10	5/10	2/10	1/10
	Isolate 6	3/10	4/10	3/10	2/10

15 ^a No. with lesions over no. examined.

20 One bird in the control group was found to have a small gizzard with an enlarged, friable intestinal tract in trial 2. The incidence of perosis was much higher in trial 2 than in the first trial, with isolates four and six producing the highest incidence of perosis (Table 11).

Table 11. Gross Skeletal Lesions

		<u>Tibial dyschondroplasia</u>	<u>Perosis</u>
5	Control	3/10 ^a	1/10
	Isolate 1	4/10	2/10
	Isolate 2	3/10	1/10
	Isolate 3	1/10	3/10
10	Isolate 4	3/10	3/10
	Isolate 5	3/10	3/10
	Isolate 6	1/10	7/10

15 ^a No. with lesions over no. examined.

Tibial dyschondroplasia was found in all of the groups including the controls in trial 2.

20 Histological lesions in the proventriculus varied from hyperplasia or hypertrophy of the glandular epithelium to fibrosis and oedema. Focal areas of necrosis were occasionally seen in the proventricular glandular epithelium. Focal myocarditis, bursal atrophy and catarrhal enteritis were
25 found in all of the injected groups.

 Histological evaluation of the femur from chicks used in trial 1 indicated that animals injected with either isolate had several lesions involving the growth plate, periosteum and ossification groove. The growth plate had
30 transverse and vertical clefts extending across the deeper portions of the proliferating and adjacent transitional zone. There was a decrease in numbers of chondrocytes along the edges of these clefts. The vertical cleft frequently
35 separated the perichondrium from the physis. Necrosis of

cartilage and fragmentation or bending of the bone bark were present at the junction of the physis with the periosteum and the periosteum was frequently hyalinized.

Similar lesions were occasionally seen in the control group. However, the lesions were much more severe and with a higher incidence in those chicks injected with isolate one.

Discussion

Proventriculitis presents a very confusing clinical picture for the diagnostician. The clinical lesions are variable and in many instances diarrhea, stunting and catarrhal enteritis are the only clinical signs presented. An enlargement of the proventriculus with a reduction in the size of the gizzard and hydropericardium were the most frequently encountered postmortem lesions seen in these trials. Chicks injected with tissue culture propagated viruses were smaller than the noninjected control chicks, exhibited an abnormal feather development and had an increase in the incidence of lameness.

Microscopic bone lesions in the growth plate, periosteum and ossification groove are of interest because femoral head necrosis is usually much more severe in flocks with proventriculitis. The vertical clefts extending across the deeper layers of the proliferating zone and adjacent transitional zones could easily be contributing to this higher incidence of femoral head necrosis and other causes of lameness in these flocks. Vertommen et al, Avian Pathol. 9:143-152 (1980), have shown an increase in alkaline phosphatase and a decrease in the plasma carotenoid level in chicks suffering from proventriculitis.

Pigmentation of the skin and shanks is a major economical consideration in some areas of the country. Considerable effort is expended to insure a deep yellow coloration of the shanks of broilers in some companies. Pale yellow shanks were a constant clinical feature in those infected chicks used in these trials.

Decreased body weights were also observed in all of the injected groups. Elevated feed conversions and decreased body weights are a constant clinical feature in chickens with proventriculitis. Although feed conversions were not
5 tabulated in any of these trials, there was a considerable amount of undigested feed in the fecal material in the injected groups.

Example 3

10

Development of a Microneutralization Test

Using viral isolate 43A or CO₈, a microneutralization test has been developed to quantify antibodies
15 against the viruses involved in the condition. The test uses constant amount of virus and the antiserum is diluted two-fold. Results obtained from the test revealed that after inoculation there is a rise in the amount of antibody present in the chickens.

20

Example 4

Protective Effects of Maternal Antibody

25 An oil based killed vaccine was produced from the CO₈ strain and evaluated in broiler breeder hens. The CO₈ reovirus was propagated in 18-24 hr chick embryo fibroblasts. After 48 hrs post infection the cells plus media were harvested. The cells were sonicated with an ultrasonic
30 generator at a setting of 8 for 1 1/2 minutes (Sonifier Cell Disruptor, Model W185, Heat Systems-ultrasonics, Inc., Plainview, L.I., NY). The virus was inactivated with 0.1% betapropiolactone for 2 hrs. After 2 hrs, 20% was blended
35 with Marcol 52, Span 80, Tween 80, Benzyl alcohol and Triethanolamine. The virus and the other

ingredients were blended for 2 to 3 minutes with a Silverson homogenizer (Silverson Machines, Ltd., Waterside, Chesham, Bucks, England). One-half ml of the vaccine was injected subcutaneously (behind the neck) into chickens between 3 and 24 weeks of age. Fifty adult Arbor Acres pullets were divided into two groups of twenty-five each and one group was vaccinated twice at four week intervals with this vaccine. Progeny from both groups were hatched and used in a challenge trial to evaluate the efficacy of this vaccine. The progeny from the vaccinated hens grew and performed as well as the non-challenged progeny. The progeny of the non-vaccinated hens were much smaller and had a higher incidence of lameness than the progeny from vaccinated hens.

15

Example 5

Field Trials in Turkeys

Twenty thousand turkey hens have been vaccinated with the CO₈-derived vaccine. Ninety thousand poults from vaccinated hens and 90,000 poults from non-vaccinated hens have been evaluated in this study. The major criteria used in this study was the mortality occurring the first two weeks of life in these flocks. Currently the mortality in the progeny from vaccinated hens is one-half of that in the progeny from the non-vaccinated flocks.

30

Antibody Response to Experimental Vaccines

An oil-emulsion, betapropiolactone inactivated vaccine has been produced from representative isolates. Geometric mean antibody titers of breeder flocks vaccinated

35

at 18-22 weeks of age with inactivated oil emulsion reovirus vaccine produced from the CO₈ strain ranged from 127.9 to 344.6 ; whereas, the geometric mean titers of unvaccinated breeder flocks ranged from 5.3 to 17.4 (Table 12).

5 Table 12. Geometric mean antibody titers of broiler breeder flocks vaccinated with inactivated oil emulsion vaccine prepared from the CO₈ strain. ^A

10

	Flock No.	No. Chickens	Vaccinated with Reovirus	Age of Flock Vaccination	Age of Flock	Geometric Mean Antibody Titers
15	1	8067	Yes	24	34	127.9
	2	8094	"	24	34	344.6
	3	8116	"	26	34	282.6
	4	8316	"	26	34	197.4
20	5	8311	"	23	31	152.2
	6	8307	"	23	31	231.9
	7	8127	"	21	29	182.9
	8	8097	"	21	29	156.0
25	9	8342	"	20	28	215.3
	10	8752	No	-	48	17.4
	11	8804	"	-	48	8.7
	12	8410	"	-	46	12.9
30	13	8515	"	-	46	5.3

^A CO₈ virus was used as the indicator virus in the microneutralization test.

In Table 13, it is shown that chickens vaccinated at one day of age with the oil emulsion vaccine responded serologically to the vaccine.

5 Table 13. Geometric mean antibody titers of broiler chickens.

	Flock No.	No. Chickens	Vaccinated With Reovirus ^A	Age of Flock	Geometric Mean ^B Antibody Titers
10	1	21,300	Yes	6 weeks	1024
	2	21,300	Yes	6 weeks	469.5
	3	21,300	Yes	5 weeks	512
	4	21,300	Yes	5 weeks	209.1
15	5	21,300	No	1 1/2 weeks	1.5
	6	21,300	Yes	1 1/2 weeks	24.7
	7	21,300	Yes	1 1/2 weeks	36.2
	8	21,300	No	1 1/2 weeks	1.5
20	9	21,300	No	3 weeks	12.2
	10	21,300	Yes	3 weeks	312.2
	11	21,300	Yes	3 weeks	608.2
	12	21,300	No ^C	9 days	63.9
25	13	21,300	No ^C	9 days	81.3
	14	21,300	No	9 days	14.7
	15	21,300	Yes	7 weeks	165.9
	16	21,300	Yes	7 weeks	98.7
30	17	21,300	Yes	7 weeks	90.5
	18	21,300	No	7 weeks	18.4

A

Inactivated, oil emulsion vaccine prepared from CO₈ strain Vaccination occurred at 1 day of age.

B

Seven-week-old chickens vaccinated subcutaneously at one day of age had a geometric mean titer that ranged from 90.5 to 165.9, while the unvaccinated chickens had a geometric mean titer of 18.4. Also of interest are the geometric mean titers of nine-day-old chickens (63.9 and 81.3) that were derived from breeder flocks which were vaccinated with the oil emulsion vaccine. In comparison nine-day-old unvaccinated chickens derived from breeder flocks which were not vaccinated had a geometric mean titer of 14.7.

Since it would be most economically feasible to vaccinate the breeder flocks with the inactivated oil emulsion vaccine, maternal antibody would have to protect the broiler chickens for at least 2-3 weeks. As shown in Table 14, maternal antibody titers of one-day-old chickens derived from breeder flocks vaccinated with the inactivated vaccine varied from 149.1 to 234.8 ; whereas, the geometric mean antibody titer of chickens from an unvaccinated breeder flock was 1.0.

Table 14. Geometric mean antibody titers of one-day-old chickens

25	Flock No.	No. Chickens	Breeder's Vaccinated	Geometric Mean ^A Antibody Titers
	1	10	Yes	234.8
	2	10	Yes	159.1
30	3	10	Yes	149.1
	4	10	No	1.0

Example 7Field Trials in Broilers

Although laboratory trials indicate that the
5 vaccine is effective in protecting chickens against the
proventriculitis virus, results from field trials give more
conclusive evidence that this vaccine is effective in
preventing the syndrome. The parameters used were total
condemnations, septicemia/toxemia, parts, average weights
10 and feed conversions. Data from 1,670,508 broiler chickens
vaccinated at one day of age generally had better
condemnations and performance than chickens not vaccinated
with the inactivated vaccines (Table 15).

Table 15. Condemnations of broiler chickens at the processing plant.

Except for C farm, test flocks were vaccinated with CO_g vaccine at 1 day of age

WK	GROWER	NO SOLD	% LIVABILITY	TOTAL COND (%)	AIR SAC COND (%)	SEP/TAX COND (%)	MAREK'S COND (%)	PARTS (%)	AV WT	FEEL CONV
1	1	60300	94.4	.85	.06	.62	.01	.85	4.85	1.97
	2	6050	94.7	.73	.07	.57	.01	.81	4.63	1.95
	3	60100	94.1	1.00	.11	.84	.01	1.14	4.74	2.01
WK AV	-	1002250	95.2	1.06	.13	.82	.01	1.04	4.59	2.03
2	1	40500	95.1	.81	.25	.97	.01	1.19	4.58	2.09
	2	61900	96.9	.74	.12	.61	0	1.29	4.32	2.03
	3	48900	93.7	.89	.13	.69	0	1.00	4.46	2.00
	4	51400	92.8	1.15	.25	.85	0	1.25	4.44	2.01
WK AV	-	996113	95.1	1.15	.18	.86	.01	1.30	4.48	2.06
3	1	19375	94.5	.45	.02	.39	0	.96	4.79	1.97
WK AV	-	941759	94.3	.76	.16	.53	.01	.98	4.61	2.07
4	1	59436	93.3	.63	.33	.29	0	1.39	4.77	2.05
WK AV	-	950694	94.4	.64	.12	.44	.01	1.45	4.68	2.07
5	1	58783	92.0	.44	.05	.34	0	.95	4.74	2.09
	2	61635	95.3	.89	.33	.49	0	1.02	4.72	2.03
WK AV	-	803803	94.2	.55	.11	.38	.01	.98	4.71	2.07
6	1	58607	91.7	.36	.05	.28	.01	.91	5.07	2.08
	2	40593	95.3	2.24	.05	1.44	0	1.18	4.27	2.03
	3	59827	93.6	.42	.06	.32	0	.86	4.80	2.01
	4	40054	94.0	.35	.09	.22	0	1.00	4.78	2.05
WK AV	-	1031316	93.7	.52	.10	.35	.01	1.11	4.80	2.08
7	1	39920	93.7	.94	.37	.56	0	1.00	4.57	2.04
	2	40107	94.2	.53	.07	.41	0	.76	4.57	2.10
	3	59347	92.9	.58	.06	.47	0	.87	4.65	2.04
	4	59821	93.6	.64	.05	.23	0	.72	4.83	2.05
WK AV	-	1013531	94.3	.75	.13	.54	.01	.94	4.54	2.08

TABLE 15. (continued)

K	GROWER	NO SOLD	% LIVABILITY	TOTAL COND (%)	AIR SAC COND (%)	SEP/TAX COND (%)	MAREK'S COND (%)	PARTS (%)	AV WT	FEED CONV
1		58943	92.2	.64	.04	.54	0	.75	4.72	1.976
2		59960	93.8	.45	.05	.36	0	.83	4.45	2.04
3		38303	89.9	.74	.15	.51	0	.91	4.54	1.99
4		47925	93.6	.86	.24	.75	0	.95	4.36	2.10
AV	-	936020	93.1	.73	.17	.57	.01	.91	4.46	2.05
1		52300	95.4	.49	.13	.31	0	.71	4.85	2.09
2		59158	92.6	.22	.04	.14	0	.56	4.60	2.11
3		63158	92.3	.36	.07	.25	0	.90	4.58	2.06
AV	-	805922	94.3	.57	.13	.35	.03	.87	4.44	2.08
1		43602	92.8	.46	.11	.29	0	.85	4.69	2.04
2		48169	94.1	.42	.07	.26	0	.96	4.64	2.04
3		58365	91.3	.90	.07	.57	0	.87	4.29	2.04
4		58284	91.2	.42	.10	.25	0	.62	4.67	2.06
AV	-	870625	93.1	.53	.14	.32	.01	.86	4.54	2.06
1		59559	93.2	.30	.24	0	0	.93	4.74	2.08
2		38677	90.8	.84	.43	.35	0	1.23	4.63	2.06
AV	-	977077	94.1	.64	.25	.39	.01	.97	4.51	2.08
1(C farm)	A	59709	94.1	.49	.13	.22	0	.75	4.82	2.03
2	"	58603	95.2	.51	.10	.31	0	.63	4.91	2.08
3	"	48433	95.4	.39	.09	.22	.01	.84	4.73	2.05
4	"	59903	95.1	.60	.11	.41	0	.79	4.69	2.07
AV	-	964331	94.2	.84	.14	.57	.02	1.13	4.70	2.10

^A Not vaccinated, but were progeny of vaccinated breeder flocks.

0101348

The unvaccinated birds were listed as the week's average or the total number of chickens processed that week. This figure does not include the vaccinated chickens. Also, the 226,642 broiler chickens designated as "C farm" chickens were progeny from breeder flocks that were vaccinated with the inactivated vaccine. However, the progeny were not vaccinated. As shown in Table 15, maternal antibody did protect these chickens. The performance and condemnation were significantly better than in the unvaccinated chickens. Also, it should be pointed out that during the "grow-out" period there were many more culls in flocks of chickens that are not vaccinated and do not come from vaccinated parent flocks when compared to progeny that are derived from vaccinated breeder flocks (Table 16).

Table 16. Comparison of mortality and cull chickens in broiler flocks derived from either vaccinated or unvaccinated breeder flocks

GROWER NO.	VACCINATED PARENT FLOCK	NO. OF BROILERS	AGE OF BROILERS	MORTALITY	CULLS
1	No	21,300	6	207	569
	No	21,300	6	231	480
	No	21,300	6	267	565
2	Yes	21,300	6	213	105
	Yes	21,300	6	243	163
	Yes	21,300	6	271	109
3	No	21,300	6	257	225
	Yes	21,300	6	230	115

Example 8Laboratory Vaccination Trials

5 The results shown in Tables 17-21 indicated that
in laboratory trials the CO₈ virus is the best candidate for
a vaccine when compared to the 81-5, 1133(VA) and 172 iso-
lates. These broiler chickens were vaccinated at 1 day of
age with inactivated oil emulsion reovirus vaccine and were
challenged intramuscularly or orally at 18 days with either
10 the 81-5 or 172 reovirus isolate. Regardless of the method
of challenge or the challenge virus used, the CO₈ vaccine
proved to be the most effective. As shown in Table 17 there was
weight depression in the control chickens when compared to
the vaccinated chickens. Although there were lesions in the
15 chickens vaccinated with 81-5, 1133(VA) and 172, the lesions
were not as severe in the vaccinated chickens as in the
controls. The weight depression was not as dramatic in the
chickens challenged orally with the 81-5 isolate. In order
to determine the immunosuppressive effects of the proventri-
20 culitis virus the chickens in Tables 17-21 were vaccinated
at 25 days with Newcastle disease virus vaccine. Although
there was probably some immunosuppression in some of the
groups, it was not significant. More time should have elap-
sed between challenging the birds with the reovirus and the
25 vaccination of the chickens with the Newcastle disease
vaccine.

Table 17. Weights and lesions after vaccination of one-day-old chickens with reovirus oil emulsion vaccine and intramuscular challenge at 18 days of age with the 81-5 isolate

5

Vaccine	Weights (Lbs)		No. +/No. Started
	Days Post Challenge		
	11	26	
CO ₈	2.46	3.96	1/15
81-5	2.43	3.86	13/15
1133(VA)	2.45	3.865	13/16
172	2.40	3.71	14/14
CONTROL	2.50	3.69	14/14

15

Table 18. Weights and lesions after vaccination of one-day-old chickens with the reovirus oil emulsion vaccine and oral challenge at 18 days of age with the 81-5 isolate

20

Vaccine	Weights (Lbs)		No. +/No. Started
	Days Post Challenge		
	11	26	
CO ₈	2.45	3.74	3/16
81-5	2.43	3.84	13/14
1133(VA)	2.33	3.71	13/15
172	2.24	3.63	10/15
CONTROL	2.33	3.65	10/15

30

Table 19. Weights and lesions after vaccination of one-day-old chickens with reovirus oil emulsion vaccines and intramuscular challenge at 18 days of age with the 172 isolate

5

Vaccine	Weights (Lbs)		No. +/No. Started
	Days Post Challenge		
	11	26	
CO ₈	2.25	3.89	3/16
81-5	2.36	3.76	10/16
1133(VA)	2.32	3.80	13/16
172	2.31	3.78	11/15
CONTROL	2.28	3.78	10/13

Table 20. Weights and lesions after vaccination of one-day-old chickens with reovirus oil emulsion vaccines and oral challenge at 18 days with the 172 isolates

20

	Vaccine	Weights (Lbs)		No. +/No. Started
		Days Post Challenge		
		11	26	
25	CO ₈	2.24	3.76	3/15
	81-5	2.40	3.77	9/12
	1133(VA)	2.38	4.02	9/15
	172	2.28	3.83	7/15
30	CONTROL	2.40	3.88	11/15

30

Table 21. Weights and lesions after vaccination of one-day-old chickens with reovirus vaccine

5	Vaccine	Weights (Lbs)		No. +/No. Started
		Days Post Challenge		
		11	26	
	CO ₈	2.39	3.70	2/15
	81-5	2.48	3.52	2/10
	1133(VA)	2.57	3.55	1/13
15	172	2.47	3.49	1/13
	CONTROL	2.48	3.51	2/13

20 Results from Table 22 clearly demonstrate that the CO₈ isolate is superior to the 81-5, 1133(VA) and 172 isolates as candidates for a vaccine. The CO₈ vaccinated chickens were protected when challenged with either the 81-5, 1133(VA) or 172 isolates. Chickens vaccinated with the other
 25 isolates (172, 1133, 81-5) were protected when challenged with its homologous challenge virus but none were as effective as the CO₈ vaccine.

Table 22. Gross lesions after vaccination of one-day-old chickens with reovirus vaccines and intramuscular challenge at 18 days of age

5	Vaccine	Challenge virus				CON	CON
		CO ₈	81-5	1133(VA)	172		
10	CO ₈	1/8 ^{A,B}	1/8	0/8	0/7		
	81/5	5-8		2/8	4/8	2/7	
	1133(VA)	2/8		7/8	2/8	2/5	
	172	2/8		6/8	3/8	0/5	
15	Control	7/8		6/8	8/8	7/8	
	Control	-	-	-	-	0/6	0/6

A No./+ No. Started

B Not including early mortality

The CO₈ isolate was deposited at the ATCC on July 7, 1982, with ATCC No. VR 2040.

Having now fully described this invention it will be apparent to those of ordinary skill in the art that the same can be performed within a wide and equivalent range of parameters, conditions, compositions, modes of administration, isolates, avian species, and the like, without affecting the spirit or scope of the invention or of any embodiment thereof.

C L A I M S

1. A vaccine capable of protecting poultry against proventriculitis.

2. The vaccine of Claim 1 which comprises antigenic material capable of inducing in said poultry an immune response against avian proventriculitis reovirus.

5

3. The vaccine of Claim 2 wherein said antigenic material is selected from the group consisting of unattenuated, attenuated or inactivated proventriculitis reovirus.

10

4. The vaccine of Claim 3 wherein said unattenuated, attenuated or inactivated reovirus has the identifying characteristics of ATCC No. VR 2040 or a progeny thereof.

5. The vaccine of Claim 2 which comprises said antigenic material in combination with a pharmacologically acceptable carrier.

15

6. The vaccine of Claim 5 wherein said carrier is a biocompatible oil.

7. The vaccine of Claim 1 which comprises live or inactivated reovirus having the identifying characteristics of ATCC No. VR 2040, or a progeny thereof together with a biocompatible oil.

20

8. The vaccine of Claim 3 wherein said reovirus is inactivated with propiolactone.

25

9. The vaccine of Claim 3 wherein said reovirus is attenuated by serial passage.

10. The vaccine of Claim 1 in unit dosage form.

11. Avian proventriculitis reovirus substantially free of cellular components.

30

12. The reovirus of Claim 11 having the identifying characteristics of ATCC No. VR 2040 or a progeny thereof.

13. The reovirus of Claim 11 having substantially no virulence.

14. The reovirus of Claim 13 wherein said lack of virulence is caused by inactivation of said reovirus.

5 15. The reovirus of Claim 11 in detectably labeled form.

16. The reovirus of Claim 15 wherein said label is a radiolabel, an enzyme label or a fluorescent label.

17. A process of preparing a vaccine capable of
10 protecting poultry against avian proventriculitis which comprises :

separating proventriculitis reovirus from reovirus-carrying tissue of a poultry animal diseased with proventriculitis ;

15 inoculating a cell culture capable of sustaining the growth and replication of said reovirus ;

extracting said virus from cellular and nonviral components, and

20 rendering said reovirus into a form suitable for administration to poultry.

18. The process of Claim 17 wherein said separation step is carried out on intestinal or visceral tissues.

19. The process of Claim 17 wherein said extraction is carried out by freeze-thawing.
25

20. The process of Claim 17 wherein said form suitable for administration to poultry is obtained by mixing said purified reovirus with a biocompatible oil.

21. The process of Claim 17 wherein said form
30 suitable for administration to poultry is obtained by inactivating said purified reovirus.

22. The process of Claim 21 wherein said inactivation is carried out with propiolactone.

35 23. The process of Claim 17 wherein said cell culture is chicken kidney embryo cells.

24. The process of Claim 17 wherein said form suitable for administration is obtained by attenuating said reovirus by passage.

25. A method of protecting a poultry animal
5 against proventriculitis which comprises :

vaccinating said poultry animal with the avian proventriculitis vaccine of Claim 1, in an amount sufficient to produce an immune response against proventriculitis reovirus in said animal.

26. The method of Claim 25 wherein said vaccine
10 comprises antigenic material selected from the group consisting of unattenuated, attenuated or inactivated proventriculitis reovirus.

27. The method of Claim 26 wherein said unattenuated, attenuated or inactivated reovirus has the identifying characteristics of ATCC No. VR 2040 or a progeny thereof.
15

28. The method of Claim 26 which comprises administering said antigenic material in combination with a pharmacologically acceptable carrier.
20

29. The method of Claim 28 wherein said carrier is a biocompatible oil.

30. The method of Claim 26 which comprises administering live or inactivated reovirus having the identifying characteristics of ATCC No. VR 2040, or a progeny thereof together with a biocompatible oil.
25

31. The method of Claim 26 wherein said reovirus is inactivated with propiolactone.

32. The method of Claim 26 wherein said reovirus
30 is attenuated by serial passage.

33. The method of Claim 25 wherein said poultry animal is a reproductive hen.

**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ BLACK BORDERS
- ☐ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☐ FADED TEXT OR DRAWING
- ☐ BLURRED OR ILLEGIBLE TEXT OR DRAWING
- ☐ SKEWED/SLANTED IMAGES
- ☒ COLOR OR BLACK AND WHITE PHOTOGRAPHS
- ☐ GRAY SCALE DOCUMENTS
- ☒ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☐ REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☐ OTHER: _____

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.